Interaction between two adapter proteins, PAG and EBP50: a possible link between membrane rafts and actin cytoskeleton

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Abstract Phosphoprotein associated with GEMs (PAG), also known as Csk-binding protein (Cbp), is a broadly expressed palmitoylated transmembrane adapter protein found in membrane rafts, also called GEMs (glycosphingolipid-enriched membrane microdomains). PAG is known to bind and activate the essential regulator of Src-family kinases, cytoplasmic protein tyrosine kinase Csk. In the present study we used the yeast 2-hybrid system to search for additional proteins which might bind to PAG. We have identified the abundant cytoplasmic adapter protein EBP50 (ezrin/radixin/moesin (ERM)-binding phosphoprotein of 50 kDa), also known as NHERF (Na^+/H^+ exchanger regulatory factor), as a specific PAG-binding partner. The interaction involves the C-terminal sequence (TRL) of PAG and N-terminal PDZ domain(s) of EBP50. As EBP50 is known to interact via its C-terminal domain with the ERM-family proteins, which in turn bind to actin cytoskeleton, the PAG–EBP50 interaction may be important for connecting membrane rafts to the actin cytoskeleton. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Raft; Microdomain; Cytoskeleton; Adapter protein; PDZ domain

1. Introduction

Phosphoprotein associated with GEMs (PAG) [1], also known as Csk-binding protein (Cbp) [2] is a broadly expressed, palmitoylated transmembrane adapter protein present in glycosphingolipid-enriched membrane microdomains (GEMs; also known as membrane rafts). PAG binds (via phosphorylated Tyr-317) the protein tyrosine kinase (PTK) Csk, a major negative regulator of Src-family kinases [1,2], and thus acts as a 'co-factor' helping to bring the cytoplasmic PTK Csk to the membrane microdomains rich in its substrates, the Src-family kinases. In addition, the formation of the PAG–Csk complex apparently increases the Csk activity also via a conformational mechanism [3]. Thus, the PAG–Csk complex suppresses the activity of membrane-associated Src-family kinases which is probably important in regulation of essential cellular functions such as growth and receptor signaling. It was demonstrated that ligation of the T-cell receptor (TCR)/CD3 complex by agonistic antibodies is accompanied by transient tyrosine dephosphorylation of PAG and release of Csk, which may help to increase the activity of Src-kinases (namely Lck and Fyn) during the early phases of T-cell activation [1].

In the present study we used the yeast 2-hybrid (Y2H) system to search for additional proteins which might interact with the cytoplasmic domain of PAG.

2. Materials and methods

2.1. Cells and transfectants

Wild-type MDCK cells (canine kidney epithelial; provided by Dr. H. Stockinger, University of Vienna) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) at 37°C in 5% CO_2. To produce stable transfected cell lines, cDNAs coding for either PAG or PAG lacking the C-terminal sequence TRL (ΔPAG) were cloned to pEFIRES expression vector [4]. These constructs were transfected into the MDCK cells using LipofectAMINE reagent (Life Technologies). Transfectants were selected for G418 resistance and cloned by limiting dilution; positive clones were selected by Western blotting and maintained in DMEM supplemented with G418 (800 μg/ml, potency > 450 μg/mg). Human adenocarcinoma Caco-2 cells (provided by Dr. L. Tučková, Institute of Microbiology AS CR, Prague, Czech Republic) were maintained in Eagle's minimum essential medium supplemented with 20% heat-inactivated FCS and 0.1 mM non-essential amino acids. Human T-cell line Jurkat and B-cell line Raji were maintained in RPMI medium containing 10% FCS. Human peripheral blood αβT-cells were prepared from buffy coats by cell sorting [1].

2.2. Reagents, antibodies and recombinant proteins

If not stated otherwise, the reagents used were from Sigma. Mouse monoclonal antibodies (mAbs) against the cytoplasmic part of human PAG were produced as described earlier [1]. Mouse mAb against ezrin–radixin–moesin (ERM)-binding phosphoprotein 50 kDa (EBP50 (EBP-10; IgG2b)) was produced by standard procedures from Balb/c mouse immunized with recombinant EBP50. Rabbit anti-serum against human EBP50 cross-reactive with the canine homolog, bacterially produced His-tagged full-length EBP50, glutathione-*S*-transferase (GST)-PDZ1 and -PDZ2 domains of EBP50 were described elsewhere [5,6]. A GST–ezrin construct (residues 1–296) was generated by polymerase chain reaction (PCR) using human kidney cDNA as a template and the PCR product was subcloned in-

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Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; DMEM, Dulbecco's modified Eagle's medium; EBP50, ERM-binding phosphoprotein 50 kDa; ERM, ezrin–radixin–moesin; FCS, fetal calf serum; GEM, glycosphingolipid-enriched membrane microdomain; PAG, phosphoprotein associated with GEMs; PBS, phosphate-buffered saline; PTK, protein tyrosine kinase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Y2H, yeast 2-hybrid system

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frame into pGEX-5x-2. The insert was verified by DNA sequencing and the construct was used for bacterial expression.

2.3. Y2H cDNA library screening for proteins interacting with PAG

The cytoplasmic part of PAG (amino acids 192–432) was subcloned into the Y2H plasmid pGBKT7 (Clontech), transformed into the yeast strain AH109 and used as a 'bait' in a Y2H screening of the human leukocyte MATCHMAKER cDNA library (Clontech) transformed in the yeast strain Y187 performed by yeast mating according to the manufacturer's instructions.

2.4. Membrane preparation

Cells (2×10^8) were resuspended in 1 ml of ice-cold hypotonic buffer (10 mM HEPES pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, 1 mM AEBSF, 10 mM EDTA), incubated on ice for 15 min and then passed 10× through the 25-gauge needle. NaF and Na_3VO_4 (10 and 1 mM final concentrations, respectively) were added after the cell disruption and the suspension was centrifuged for 5 min at $400\times g$ and 0°C to remove nuclei. The ice-cold post-nuclear supernatants were centrifuged for 10 min at $18\,000\times g$ and 0°C to pellet the membranes.

2.5. Immunoprecipitation experiments

Cells or membranes were solubilized in isotonic lysis buffer (Tris-buffered saline pH 8.2, containing 1 mM AEBSF, 10 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4 and 1% detergent laurylmaltoside (*n*-dodecyl β-D-maltoside; Calbiochem)) and post-nuclear supernatants were used for immunoprecipitations essentially as described before [1] using Protein A-purified mAbs covalently coupled to CNBr–Sepharose beads (AP Biotech) as immunosorbents. Immunoisolation was performed using minicolumns (50 μl packed volume) of immunosorbent. Post-nuclear cell or membrane lysates were passed through the columns; after washing with 10 column volumes of the lysis buffer, bound proteins were eluted with 2 column volumes of 2× concentrated non-reducing Laemmli sample buffer and the flow-through and eluted fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western blotting. In some experiments, GST-tagged ezrin bound to Glutathione Sepharose 4B (AP Biotech) was used for an affinity isolation of EBP50. In this case minicolumns packed with 50 μl of Glutathione Sepharose 4B either loaded with 2 μg of recombinant GST–ezrin or unloaded were used to isolate EBP50 from the membrane lysate as described above.

2.6. Ligand blotting

PAG immunoprecipitates were subjected to SDS–PAGE followed by electroblotting onto PVDF membrane and standard immunoperoxidase staining with rabbit anti-EBP50 anti-serum. The blot was then reprobed with the recombinant EBP50 PDZ1 or PDZ2 domains (20 μg/ml in phosphate-buffered saline (PBS), 0.05% Tween 20, 0.5% non-fat dry milk) followed by rabbit anti-EBP50 anti-serum and horseradish peroxidase-conjugated secondary antibody.

2.7. Fractionation of detergent-resistant GEMs (membrane rafts) by density gradient ultracentrifugation

Cells (5×10^6 Jurkat cells or MDCK cells obtained from one confluent 10-cm culture dish) were washed in PBS, resuspended in 500 μl of ice-cold lysis buffer containing 3% detergent Brij-58 and lysed 30 min on ice. The lysate was mixed 1:1 with 80% (w/v) sucrose in lysis buffer and placed at the bottom of a 5.2 ml polyallomer centrifuge tube (Beckman Instruments), then carefully overlaid with 3.5 ml of 30% (w/v) sucrose in lysis buffer and finally with 1 ml of lysis buffer. Centrifugation was performed at 2°C in Beckman SW55Ti rotor (18 h, 52 000 rpm). Eight 0.63 ml fractions were collected gradually from the top of the gradient, proteins were separated by SDS–PAGE and analyzed by immunoblotting.

3. Results

3.1. Identification of EBP50 as a potential binding partner of PAG by Y2H

The Y2H screening of human leukocyte cDNA library was performed using the c-terminal part of the cytoplasmic domain of PAG (amino acids 192–432) as a 'bait'. Out of approximately 2 million transformants, roughly 200 colonies grew in the selective media lacking adenosine and histidine and were positive in the β-galactosidase assay. Among these, more than 70 clones contained various parts of EBP50, also known as NHERF (Na^+/H^+ exchanger regulatory factor) [7,8]. EBP50 did not interact with the control bait pGBKT7-lamin or with the empty vector pGBKT7, documenting the specificity of its interaction with PAG (not shown). EBP50 is an abundant cytoplasmic protein containing two N-terminal PDZ domains and a C-terminal domain which is known to bind to the ERM-family proteins [8]. The PDZ1 domain of EBP50 was previously demonstrated to bind to the C-terminal Thr-Arg-Leu-COOH (TRL) motif of several membrane proteins including the cystic fibrosis transmembrane conductance regulator (CFTR) [6,9,10]; this interaction may target CFTR to the apical surface of epithelial cells [11]. Importantly, the C-terminal TRL motif is also present in PAG, thus rationalizing the result of the Y2H screening.

3.2. The PAG–EBP50 interaction occurs in mammalian cells

MDCK cells transfected with human PAG or PAG lacking the C-terminal TRL sequence (ΔPAG) were solubilized in a solution of 1% laurylmaltoside, a detergent disrupting GEMs, and the lysates were subjected to immunoprecipitation on an anti-PAG immunosorbent. Western blotting analysis of the immunoprecipitates demonstrated co-precipitation of endogenous EBP50 with PAG from the transfectants containing the wild-type human PAG but not ΔPAG (Fig. 1A). Similarly, a specific co-precipitation of EBP50 with endogenous PAG could be demonstrated from detergent lysates of peripheral blood T-cells, wild-type Raji, Jurkat and Caco-2 cells (Fig.

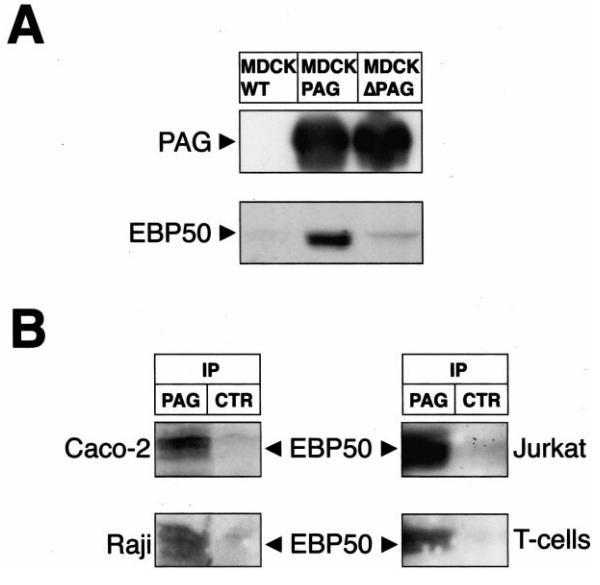

Fig. 1. Co-precipitation of EBP50 with PAG. A: Wild-type MDCK cells (MDCK WT) were stably transfected with human PAG (MDCK PAG) or PAG lacking the last three C-terminal amino acids (MDCK ΔPAG). Detergent (1% laurylmaltoside) lysates of these cells were immunoprecipitated on an anti-PAG immunosorbent and the immunoprecipitates were analyzed by immunoblotting. mAb MEM-255 and rabbit anti-serum to EBP50 were used to detect human PAG and endogenous EBP50, respectively. B: Immunoprecipitation (IP) of detergent lysates of the indicated human cells was performed on anti-PAG or irrelevant control (CTR) immunosorbents and the specifically co-precipitated EBP50 was detected as in A.

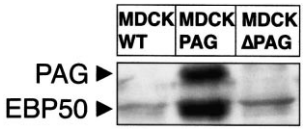

Fig. 2. Demonstration of direct interaction between PAG and EBP50 PDZ1 domain by ligand blotting. The same blots as shown in Fig. 1A were incubated with recombinant GST-PDZ1 domain; the zones containing EBP50 or the PDZ1 domain were then visualized by anti-EBP50 immunoperoxidase staining. Thus, the 50 kDa zone corresponding to the co-precipitated EBP50 was immunostained directly, while the zone corresponding by size exactly to PAG was visualized due to the binding of the PDZ1 domain to the membrane-immobilized PAG.

1B). The membrane with the electroblotted PAG or ΔPAG immunoprecipitates obtained from the MDCK transfectants was further used to demonstrate direct binding of EBP50 PDZ1 domain to PAG using the ligand-blotting approach (Fig. 2). The same results were obtained when PDZ2 domain was used (not shown); this would indicate that PAG may interact with EBP50 through any of the two PDZ domains. The results of quantitative immunoisolation of PAG indicated that only a minor fraction of EBP50 is associated with PAG (not shown). Specific interaction between PAG and EBP50 could also be demonstrated by a reciprocal co-precipitation of PAG from detergent solubilized Raji cell membranes on an anti-EBP50 immunosorbent (Fig. 3A); a similar result was also obtained when immobilized GST–ezrin was used to affinity isolate EBP50 from detergent solubilized Jurkat cell membranes: as expected, all EBP50 present in this lysate was bound to immobilized ezrin and a minor fraction of total PAG was co-isolated under these conditions (Fig. 3B).

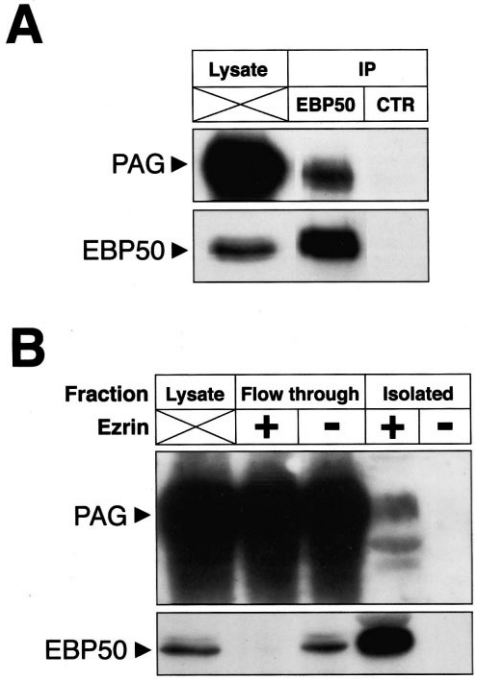

Fig. 3. Co-precipitation of PAG on anti-EBP50 immunosorbent and on immobilized ezrin. A: Detergent lysate of Raji cell membranes was immunoprecipitated on an anti-EBP50 immunosorbent (EBP50) or immobilized irrelevant control mAb (CTR). The original lysate (Lysate) and immunoprecipitates (IP) were analyzed by SDS–PAGE and immunoblotting as in Fig. 1A. B: Detergent lysate of Jurkat cell membranes was used for affinity isolation of EBP50 on immobilized GST–ezrin (+) and control glutathione–Sepharose (−). The original lysate (Lysate), materials passed through the sorbent minicolumns (Flow through) and materials eluted from the sorbents (Isolated) were analyzed by SDS–PAGE and immunoblotting as in Fig. 1A.

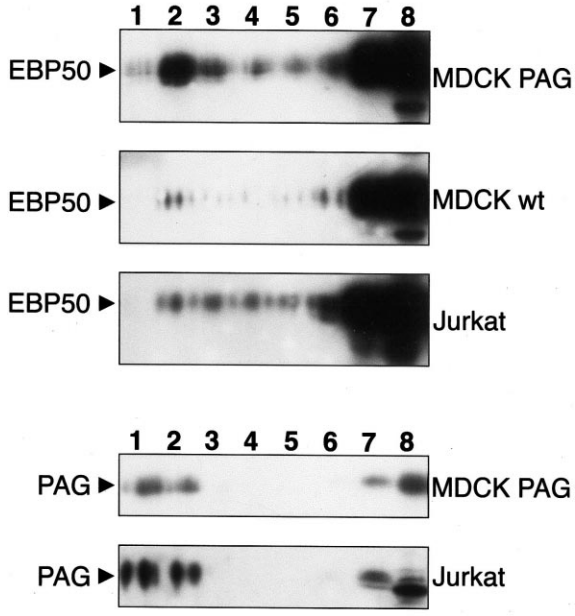

Fig. 4. The presence of EBP50 in GEMs (membrane rafts). MDCK cells transfected with human PAG (MDCK PAG), wild-type MDCK cells (MDCK wt) or Jurkat cells were detergent solubilized and fractionated by density gradient ultracentrifugation as described in Section 2.7. The fractions (numbered from the top to the bottom of the gradient) were analyzed by SDS–PAGE and immunoblotting as in Fig. 1A.

3.3. *A fraction of EBP50 is present in GEMs*

A major fraction of PAG is present in GEMs, buoyant microdomains enriched in glycosphingolipids and cholesterol, resistant to solubilization by detergents such as Triton X-100, NP40 or Brij-58 [1,2]. Therefore, we examined whether a fraction of EBP50, which has no known structural features targeting it to GEMs (such as the lipid modifications present in PAG or in Src-family PTKs) can be, due to its interaction with PAG, also detected in such large, buoyant, detergent-resistant complexes. Indeed, a markedly larger fraction of EBP50 could be detected in the typical GEMs-containing sucrose density gradient ultracentrifugation fractions of MDCK cells transfected with human PAG as compared to wild-type MDCK cells; a minor fraction of EBP50 could be demonstrated also in the GEMs of wild-type Jurkat cells (Fig. 4).

4. Discussion

Our data demonstrate that, in addition to Csk, EBP50 is another protein interacting with the transmembrane adapter protein PAG. This interaction, dependent on the PAG C-terminal motif suitable for binding to the PDZ domains of EBP50, was detected first by means of the Y2H system and was subsequently confirmed by co-isolation from detergent lysates of mammalian cells and also by ligand blotting.

PAG is a characteristic component of GEMs, the membrane structures known to be important in immunoreceptor

signaling. GEMs redistribute to the site of contact between an antigen-presenting cell and a T-cell; the interaction between activated TCR and GEMs, which is crucial for initiation of the signaling cascades, is accompanied or perhaps even caused by actin cytoskeleton redistribution [12]. Similarly, functionally important interactions between the high affinity Fcε-receptor and lipid rafts on activated mastocytes are dependent on actin cytoskeleton [13]. Thus, it may be hypothesized that the complex formed between PAG and EBP50 may help to link GEMs to F-actin (via the ERM proteins), which might be essential for redistribution of GEMs during immunoreceptor signaling.

Our data suggest that only a minor fraction of total PAG is associated with EBP50 and vice versa. A simple reason for this could be a relatively low affinity of this interaction which may cause a rapid dissociation of the complex following cell lysis and during immunoisolation; if so, our results would simply underestimate the real amount of the complex in vivo. The relatively low affinity of the PAG–EBP50 interaction could be due to a suboptimal structure of the PAG C-terminus for binding to the EBP50 PDZ domains. Although the minimal consensus sequence for binding to both PDZ1 and PDZ2 domains of EBP50 is S/T-x-L, the nature of the amino acid at the −4 position may also play a role [9,10]. Another possibility for the observed low stoichiometry of the PAG–EBP50 interaction is that major fractions of PAG or EBP50 (or both) are in vivo modified in a way interfering with the interaction. Such modifications might include, for example, phosphorylation of the threonine residue within the TRL sequence proteolytic truncation of PAG at the C-terminus or folding of native PAG molecule such that the C-terminus is not accessible for interaction with the PDZ domains. However, these modifications seem unlikely because our mAbs specifically reactive with the unmodified C-terminal peptide of PAG immunoprecipitate PAG essentially quantitatively (unpublished results). Also, a covalent modification or a conformational arrangement of a major fraction of EBP50 might prevent its binding to PAG.

In any case, even the small amounts of the PAG–EBP50 complex (compared to the total amounts of PAG and EBP50 in the cells) may be sufficient to link GEMs (membrane rafts) to the cytoskeleton. We are currently examining possible quantitative changes in the PAG–EBP50 complex induced by various cell activation treatments potentially inducing its link to ezrin-family proteins and F-actin.

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